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The results of this study clearly show that single cell techniques can be used to characterize stimulus-secretion coupling. The requirement for lower numbers of cells with these techniques means that chromaffin cells from rodents can be routinely employed. This can be advantageous to minimize biological variability which occurs with organs obtained from slaughter houses and enables the investigation of genetically-altered animals.

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Comparison of Cytosolic Ca^{2+} and Exocytosis Responses from Single Rat and Bovine Chromaffin Cells

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Abbreviations: BSA: bovine serum albumin, Ca^{2+}_{tot}: integrated Ca^{2+} response, CA: catecholamine, CA_{tot}: total CA secretion, CICR: calcium-induced calcium release, DMEM/F12: Dulbeccos' modified Eagle's/Ham's F-12, DMPP: 1,1-dimethyl-4-phenylpiperazinium, EGTA: ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetate, IP_3: 1,4,5 inositol trisphosphate

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ABSTRACT

The relationship between cytosolic Ca^{2+} and catecholamine secretion during stimulus-secretion coupling has been examined at individual chromaffin cells isolated from the cow and rat. Vesicular catecholamine exocytosis was determined via amperometric measurements with carbon fiber microelectrodes and fura-2 was used for simultaneous fluorescent monitoring of cytosolic Ca^{2+} at the same cell.[12] Individual secretory vesicles in cells from the two species were found to release similar amounts of catecholamine. In addition, the time courses for secretion from individual vesicles was similar with rat and bovine chromaffin cells. The total quantity of catecholamine released and the change in cytosolic Ca^{2+} were also similar in response to exposure to K^+ (60 mM), DMPP (50 μM), and histamine (50 μM), although both responses were more prolonged following DMPP and histamine at bovine cells. Agents that mobilize intracellular Ca^{2+}-stores such as methacholine, caffeine and bradykinin resulted in different cytosolic Ca^{2+} and exocytosis responses at the rat and bovine chromaffin cells. Results indicates a heightened Ca^{2+} store activity or a more filled state in chromaffin cells from the rat.

The results of this study clearly show that single cell techniques can be used to characterize stimulus-secretion coupling. The requirement for lower numbers of cells with these techniques means that chromaffin cells from rodents can be routinely employed. This can be advantageous to minimize biological variability[21] which occurs with organs obtained from slaughter houses and enables the investigation of genetically-altered animals.

Key Words: amperometry, chromaffin cells, catecholamine, exocytosis, fura-2, calcium ions

Because of their high yield and similarity to neurons, primary cultures of bovine chromaffin cells have been extensively employed for studies of catecholamine (CA) secretion and its regulation by cytosolic Ca^{2+}. The advent of techniques to measure chemical changes at the level of individual cells has removed the necessity of large culture yields so that now small laboratory animal models such as the rat can be used.[9,23,44] The techniques include patch clamp electrophysiology to examine ion channel activity and changes in cell capacitance[32], selective fluorescent indicators to measure intracellular ion concentrations,[14,37] and electrochemistry to monitor single exocytotic events.[8,9,20,41,44] Thus, the variability which exists in tissue from slaughter houses[21] can be avoided, and specific strains of laboratory animals, including those genetically altered, can be selectively employed.

It has been documented that chromaffin cells from different animal species exhibit notable differences in response to secretagogues. For example, stimulation of the muscarinic receptor evokes robust release in chromaffin cell populations or adrenal glands from the cat,[11] guinea pig,[31] rat,[9,39] and chicken,[19] but less so from the cow.[5] More recently, oscillations of cytosolic Ca^{2+} in response to K^+ and bradykinin have been reported that appear to be unique to chromaffin cells from rat.[10,22,23] The detection of Ca^{2+} oscillations in excitable cells also raises interesting questions about the role of Ca^{2+} in catecholamine secretion[22,40] and its species to species variation.

The present study is the first to compare cytosolic free Ca^{2+} concentration and CA secretion during exocytosis from single rat and bovine chromaffin cells. Simultaneous, single-cell measurements were made of fura-2 fluorescence and CA exocytosis with carbon-fiber microelectrodes. The effects of stimuli which induce cell depolarization as well as those which release Ca^{2+} from internal stores have been examined. Differences between the rat and bovine chromaffin cells were found and indicate significant variations in the receptor-mediated stimulus-secretion coupling from species to species. Results from direct membrane depolarization with high K^+ reveal similar maximal Ca^{2+} entry and total CA secretion (CA_{tot}) in rat and bovine

chromaffin cells. We also find that individual catecholamine vesicles from rat and cow release similar amounts of CA with similar kinetics. However, some receptor-mediated agents (1,1-dimethyl-4-phenylpiperazinium (DMPP) and histamine) lead to a longer lasting Ca^{2+} and CA responses at bovine cells than at rat cells. These prolonged effects suggest differences in the two species' receptor affinity or time for desensitization, or a different cell membrane microenvironment for the targeted receptors. Experiments with the intracellular Ca^{2+} store releasing agents methacholine, caffeine, and bradykinin suggest that the stores in rat cells are more readily accessible or contain larger amounts of Ca^{2+}.

EXPERIMENTAL PROCEDURES

Chromaffin cell cultures. Bovine adrenal chromaffin cells, enriched in epinephrine using a single-step Renografin gradient,[26] were prepared from fresh tissue as previously described.[20] Single cells were plated on glass coverslips (Carolina Biological Supply, Burlington, NC) at a density of 6×10^5 cells per 35-mm diameter plate. Rat adrenal glands were obtained from 10-15 Sprague-Dawley male rats (300-400 g) which were killed by suffocation in a CO_2 atmosphere. The abdomen was opened and adrenal medullary tissue was dissected by pressure decapsulation and immediately placed in ice cold buffer containing 150 mM NaCl, 5 mM KCl, 5 mM glucose, and 10 mM HEPES at pH 7.4. The tissue was transferred to an identical buffer containing bovine serum albumin (BSA, 0.3%) and collagenase (0.15%) for 30 min at 37°C. The tissue/collagenase mixture was repeatedly agitated with a transfer pipette to facilitate digestion. Next, the tissue was centrifuged for 15 min at 700 g, washed twice with large volumes of buffer, filtered through a 250 μm nylon mesh, and resuspended in 3 mL of 15% Renografin. An epinephrine-enriched population of cells was obtained by discontinuous Renografin gradient[26] at 7700 g for 10 min. The intermediate band was collected for experiments and plated on glass coverslips.

Both types of chromaffin cells were incubated at 37°C in a 5% CO_2 atmosphere and used at room temperature between days 3 and 8 of culture. Only 25% of the rat cells appeared to have an intact membrane whereas 90% of the bovine cells were intact. These cells showed an elevation in cytosolic Ca^{2+} and secreted catecholamine in response to K^+-exposure. The reported results are from this population of cells.

Electrochemical detection of catecholamine. Microelectrodes for the detection of catecholamine release were prepared with individual carbon fibers (5-μm radius, Thornell P-55, Amoco Corp., Greenville, SC) sealed into glass micropipettes with epoxy (Epon 828 Resin and *m*-phenylenediamine hardener, Miller-Stephenson, Danbury, CT).[16] A micropipette beveller

(Model BV-10, Sutter Instruments, Novato, CA) was used to polish the tips. The electrode tips were soaked in 2-propanol for at least 15 min before use. Electrodes were calibrated with a flow-injection apparatus using 50 μM epinephrine.[35]

For all experiments, the culture medium was replaced with Krebs-Ringer buffer containing 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM HEPES, and 2 mM $CaCl_2$, adjusted to pH 7.4 with NaOH. To monitor effects of intracellular Ca^{2+} stores, $CaCl_2$ was omitted from the buffer and 0.2 mM EGTA was added to reduce extracellular free Ca^{2+} to < 10^{-8} M.[15] Experiments were performed on the stage of an inverted microscope (Axiovert 35, Zeiss, Thornwood, NY) equipped with a fura-2 fluorescence accessory (EMPIX Imaging, Mississauga, Canada) (Fig. 1). Secretagogues were applied transiently (3-5 s) from a micropipette using pressure ejection (Picospritzer, General Valve Corp., Fairfield, NJ). The cells were exposed to 60 mM K^+ before and after each secretory agent to provide a reference response. When several concentrations of DMPP were tested at one cell, this step was omitted. In the potassium pipette solution, the concentration of NaCl in the pipette was reduced to maintain isotonicity. Carbon-fiber microelectrodes were used in the amperometric mode ($E_{applied}$ = +650 mV vs. saline-saturated calomel electrode) and positioned 1 μm away from the cell as previously described.[34] Amperometric electrode responses were measured with the EI-400 potentiostat (Ensman Instruments, Bloomington, IN), low pass filtered at 16.67 kHz, digitized and recorded on 1/2" videotape.

To quantitate exocytosis from individual vesicles, prerecorded amperometric currents were filtered at 400 Hz and digitized at 1 ms/point (Cyberamp and Axotape, Axon, Foster City, CA). Individual current spike areas (Q), amplitudes (i_{max}), and widths at half-height ($t_{1/2}$) were determined using locally written software as described previously.[34] The spike areas (units of charge) are directly proportional to the total number of moles of catecholamine detected using Faraday's law.[12] To quantitate the total amount of secretion from a stimulation, data was low pass filtered at 25 Hz and computer digitized at 20 ms/pt using a NIC-310 oscilloscope (Nicolet

Instrument Corp., Madison, WI). The areas under the entire secretion curve were measured for 60 s following secretagogue delivery and are expressed as CA_{tot}. Data are reported as mean ± s.e.m. The Mann-Whitney test (Systat, Evanston, IL) for non-parametric data was used to determine significance at the $p < .05$ level.

Fura-2 Ca^{2+} measurements. Chromaffin cells were incubated in Krebs-Ringer buffer containing 1 μM fura-2 AM (167 μM stock solution dissolved in 20% Pluronic F-127 in DMSO), 0.1% BSA, and 2 mM Ca^{2+} for 30-40 min at room temperature. After loading, the cells were rinsed twice and placed in the desired Krebs-Ringer buffer for 20 min before experiments.

A filter wheel was used to alternately excite cells at 340 nm and 380 nm (Fig. 1). A

(Insert Fig. 1 here)

shutter, limiting illumination of the sample to 40 ms every 250 ms, and a 0.5 neutral density filter were used to minimize photobleaching. Emitted fluorescence was collected through a 40 x oil-immersion objective (NA = 1.3, Fluar 40 X, Zeiss, Thornwood, NY) and passed through a 43-μm pinhole aperture to restrict the measurement area to a single cell.[15] Fluorescence from each wavelength was monitored with a photomultiplier tube (R928, Hamamatsu, Bridgewater, NJ).

The microelectrode assembly was found to have some autofluorescence (A_e), due to the *m*-phenylenediamine epoxy hardener at the tip, and the glass-encasement reflected (R_e) both excitation wavelengths (reflectance greater at 380 nm than at 340 nm) back onto the cell. Therefore, it was necessary to correct the measured fluorescence intensities at each cell for these signals by determining four control values at each excitation wavelength:

$$F_{m1} = (R_e \times F) + A_e \qquad \text{(Eq. 1)}$$

$$F_{m2} = F \qquad \text{(Eq. 2)}$$

$$F_{m3} = R_e (F + F_s) + A_e \qquad \text{(Eq. 3)}$$

$$F_{m4} = F + F_s \qquad \text{(Eq. 4)}$$

where F_{m1} describes the measured value with the electrode positioned next to a resting cell, F_{m2} is the measurement at a resting cell without an electrode, F_{m3} is the measurement with electrode positioned next to the cell at the maximal value during stimulation, and F_{m4} is the measurement at maximal value during stimulation without the electrode in place. F is the true basal cell fluorescence without electrode artifacts and F_s is the actual change in fluorescence induced by the stimulation. Solving the above equations one obtains:

$$R_e = (F_{m1}-F_{m3}) / (F_{m2}-F_{m4}) \qquad \text{(Eq. 5)}$$

$$A_e = F_{m1} - (F_{m2} \times R_e) \qquad \text{(Eq. 6)}$$

With the electrode positioned to the side of the cell, the values of R_e for each wavelength were typically close to 1, but values of A_e varied greatly with electrode tip size and proximity to the cell. Each measured fluorescence (F_m) was then corrected for electrode reflectance and autofluorescence independently according to the following equations:

$$F_{340} = (F_{m340} - A_{e340}) / R_{e340} \qquad \text{(Eq.7)}$$

$$F_{380} = (F_{m380} - A_{e380}) / R_{e380} \qquad \text{(Eq.8)}$$

The corrected fluorescence values were ratioed (F_{340}/F_{380}) and estimates of intracellular Ca^{2+} concentration were calculated using a previously published method.[12,14]

Materials--Fura-2 AM, fura-2 free acid, and Pluronic-F127 were obtained from Molecular Probes (Eugene, OR). Culture medium, Dulbecco's modified Eagle's/Ham's F-12 medium (DMEM/F12), was obtained from Gibco Laboratories (Grand Island, NY). Collagenase (Type I) for digestion of gland tissue was obtained from Worthington Biochemical (Freehold, NJ). Renografin-60 was purchased from Squibb Diagnostics (New Brunswick, NJ). All other chemicals were obtained from Sigma (St. Louis, MO), and solutions were prepared with doubly distilled water.

RESULTS

Similarities in secretion and cytosolic Ca^{2+} in response to 60 mM K^+. In single chromaffin cells loaded with fura-2, a 3-s exposure to depolarizing stimuli results in an elevation of cytosolic Ca^{2+} at both cell types (Fig. 2). The initial Ca^{2+} rise is rapid and occurs concomitantly with the onset of secretion; the subsequent decrease in cytosolic Ca^{2+} is accompanied by a cessation of release.[12] When transiently exposed to 60 mM K^+, the maximal observed $[Ca^{2+}]$ is lower in rat cells than in bovine cells (202 ± 23 nM versus 324 ± 24 nM). However, the total amount of catecholamine secretion (CA_{tot}) resulting from the K^+ exposure is similar at rat and bovine cells (0.99 ± 0.21 fmol versus 1.11 ± 0.11 fmol). Both the duration of the increase in cytosolic Ca^{2+} at half maximal response (10 ± .62 s versus 9.1 ± .33 s) and the total duration of catecholamine release (24 ± 2.1 s versus 39 ± 1.4 s) are also similar in rat and bovine chromaffin cells (Fig. 2).

(Insert Fig. 2 here)

The values of CA_{tot} and integrated area under the Ca^{2+} trace (Ca^{2+}_{tot}) induced by several other secretagogues are summarized in Table 1 as a percentage of the changes induced by 60 mM K^+. Note that Ca^{2+}_{tot} reflects both peak Ca^{2+} concentration and duration of the elevated state which allows for comparison among secretagogues.

Prolonged responses of bovine cells to DMPP. Prior work has shown that 3-s exposure of individual bovine chromaffin cells to nicotinic agonists induces exocytotic release of catecholamine.[12,20] As with bovine cells,[12] both K^+ and DMPP responses at rat cells are concentration dependent and responses are longer-lasting when induced by DMPP (3 s) than by K^+ (3 s) (data not shown). Exposure of rat chromaffin cells to the nicotinic agonist DMPP (10 μM) produced exocytotic secretion for a shorter time period than at bovine cells (34 ± 2.3 s versus 70 ± 4.9 s). This resulted in rat cells secreting less than 50% of the CA_{tot} from bovine cells (Fig. 2

and Table 1) and in turn, a smaller number of individual secretory events for 10 stimulations at 7 rat cells (438) than at 7 bovine cells (817). Although maximal Ca^{2+} values are similar (180 ± 17 nM versus 163 ± 7.1 nM) in the rat and cow, the Ca^{2+}_{tot} was greater in the cow because of the longer response duration (Fig. 2 and Table 1).

Characteristics of individual exocytotic release events. To examine the characteristics of the individual exocytotic events recorded by amperometry, cells of each type were exposed to 10 μM DMPP (3 s) every 2 min for 8 exposures. Previous studies at bovine cells have shown that the choice of secretagogue affects frequency, but not the attributes, of individual spikes.[41] The areas of individual spikes from rat and bovine cells are plotted as histograms in Fig. 3. The

(Insert Fig. 3 here)

charge histograms and mean charge values, that are proportional to the quantity of catecholamine released,[41] for the two data sets are not significantly different from one another. Mean widths at half height and maximal currents for the two species only differed by ~ 4% and 10%, respectively (data not shown), neither of which are statistically significant.

Histamine-induced responses from rat and bovine chromaffin cells. Histamine (50 μM), which promotes secretion via the H_1 receptor,[27,30,36] was transiently (5 s) delivered to individual cells in the presence of 2 mM external Ca^{2+}. High K^+ was applied before and after histamine challenges to ensure cell viability and for comparison with histamine-induced responses. At rat cells in the presence of external Ca^{2+}, the first exposure to 50 μM histamine elicited about 55% of both the catecholamine release and maximal cytosolic Ca^{2+} concentration found in the preceding K^+ exposure (n = 5 cells) (Table 1). At bovine cells the integrated Ca^{2+}_{tot} and CA_{tot} was greater than at rat cells (Table 1 and Fig. 4). The prolonged response of histamine at bovine cells resulted in secretion approximately equal to that from K^+ at the same cells (Table 1 and Fig. 4).

(Insert Fig. 4 here)

Repeated histamine deliveries (every 2 min) to rat cells resulted in decreasing maximal cytosolic Ca^{2+} concentrations and total CA secretion. After 6-12 exposures to histamine the cells no longer responded to histamine but a subsequent exposure to 60 mM K^+ caused an increase in cytosolic Ca^{2+} accompanied by exocytotic secretion.

In the absence of external Ca^{2+} (0.2 mM EGTA), rat cells gave few or no catecholamine spikes and only insignificant Ca^{2+} responses (< 10% of K^+ control) from exposure to 50 μM histamine (n = 5 cells) (Fig. 5B). However, in 50% of the bovine cells studied (n = 18), histamine was capable of eliciting significant (both values > 10% of K^+ control) catecholamine exocytosis and Ca^{2+} responses when extracellular Ca^{2+} was absent (Fig. 4).

Methacholine-induced responses at rat and bovine chromaffin cells in the presence or absence of extracellular Ca^{2+}. The muscarinic agonist methacholine (50 μM) was applied for 5 s via pressure ejection to single chromaffin cells. Data obtained during prior and subsequent exposures to 60 mM K^+ (5 s) served as a control. In the presence of extracellular Ca^{2+}, all rat cells exposed to methacholine showed significant secretion of catecholamine and rise in cytosolic Ca^{2+} (Fig. 5). The duration of both responses were on the order of those obtained with

(Insert Fig. 5 here)

60 mM K^+. Bovine cells exposed to methacholine gave more varied results. Most of the bovine cells tested resulted in a rise in Ca^{2+} but showed little or no catecholamine secretion (n = 8) (Fig. 5B), others had a large enough Ca^{2+} transient to induce significant release (n = 5) (Fig. 5B inset), and the remaining lacked any secretion or Ca^{2+} responses even though the K^+ controls were positive (n = 4). Mean CA_{tot} and the observed maximal $[Ca^{2+}]$ for the 5 bovine cells with significant responses are 32 ± 7.5 % and 54 ± 4.0 % of results from K^+ exposure. As with

histamine, repetitive application of methacholine lead to decreased responses of secretion and cytosolic Ca^{2+}.

In the absence of extracellular Ca^{2+}, most of the rat cells (n = 5 of 7) yielded a small rise in Ca^{2+} (~ 25 nM) and a few catecholamine spikes (Fig. 6C). The remaining two rat and all of the bovine (n = 9) cells did not secrete any catecholamine despite the small rise in cytosolic Ca^{2+} (Fig. 5D).

Caffeine-induced responses at rat chromaffin cells without extracellular Ca^{2+}. Caffeine was applied to single cells in the absence of extracellular Ca^{2+} to examine depletion of the homogeneously distributed caffeine-sensitive Ca^{2+} stores.[6,12] Transient exposure to 40 mM caffeine was toxic to all rat cells studied (n = 8) and 10 mM caffeine was toxic to 50 % of rat cells studied (n = 4). Cell toxicity was determined by massive catecholamine release that was in the form of a large, broad peak (or sometimes one large spike) accompanied by a rapid, large decrease in fluorescence at both excitation wavelengths, indicative of dye expulsion from the cell. These concentrations have been used with bovine chromaffin cells without adverse effects.[4,12] A dose of 1 mM caffeine did not cause toxicity at rat cells, and 50 % were found to release CA with an accompanying increase in cytosolic Ca^{2+} (n = 8 cells). In all cases of caffeine-induced CA release in media without extracellular Ca^{2+}, release only occured on the first exposure to caffeine. This was also the case with bovine cells exposed to 10 mM or 40 mM caffeine in the absence of extracellular Ca^{2+}.[12]

Oscillations of cytosolic Ca^{2+} and catecholamine release in rat chromaffin cells. To study the effects of the nonapeptide bradykinin on secretion and Ca^{2+} responses, a micropipette (tip diameter > 30 μm) containing 200 nM bradykinin was placed within 20 μm of a single rat chromaffin cell. However, before pressure ejection, leakage of bradykinin from the pipette was sufficient to induce oscillations in baseline Ca^{2+} and exocytosis in 11 of the 13 rat cells examined in Ca^{2+}-containing medium. The frequency and duration of these Ca^{2+} oscillations were found to vary from cell to cell (Fig. 6). Catecholamine secretion mimicked the Ca^{2+} patterns once the

(Insert Fig. 6 here)

cell's threshold intracellular Ca^{2+} concentration was surpassed. Fluctuations would typically begin within 60 s of positioning the micropipette and would cease within 60 s of removing the bradykinin leakage source. Transient application of 60 mM K^+ to an oscillating cell caused an increase in cytosolic Ca^{2+} and CA release without oscillations. However, the oscillations resumed ~ 40 s following K^+ application. In rat cells without fura-2, oscillations in CA secretion were still observed. Rhythmic oscillations were not observed when Ca^{2+} was absent from the external media (0.2 mM EGTA). Oscillatory responses were never observed in bovine chromaffin cells exposed to the same conditions with or without fura-2.[12] Spontaneous oscillations of basal cytosolic Ca^{2+} levels or CA secretion were not observed in resting rat or bovine chromaffin cells.

DISCUSSION

Ca^{2+}-dependent, vesicular release from rat chromaffin cells is similar to that found at bovine cells. Amperometric measurement of catecholamine release from chromaffin cells provides a way to measure the time course and quantity of the extrusion of the vesicular contents during exocytosis. Recordings from the amperometric electrode report from the surface area of the cell which is directly beneath the electrode.[35] In prior investigations at bovine chromaffin cells we have shown that the mean quantity released per exocytotic event corresponds to approximately 4 million catecholamine molecules.[41] The released quantity from multiple spikes has a distribution that is skewed to the right.[15,34,42] An identical mean and distribution is obtained in this work at rat chromaffin cells. In addition, the time course for extrusion of the catecholamines from rat and bovine chromaffin cell vesicles is found to be identical. Prior work suggests that this time course is due to the time required for dissociation of the vesicular contents following vesicle exocytosis.[15,42]

Fura-2 measurements of whole cell cytosolic calcium in bovine cells show that elevated cytosolic Ca^{2+} concentrations are a necessary condition to observe exocytosis, and that a threshold level of cytosolic free Ca^{2+} must be exceeded before exocytosis can occur.[12] In a qualitative sense, the findings in chromaffin cells from the rat are similar. Agents which produce membrane depolarization (high K^+ or activation of nicotinic receptors)[4,17,18] allow Ca^{2+} entry at both types of cells which is accompanied by exocytosis. Thus, the biochemical machinery which regulates stimulus-secretion coupling at chromaffin cells appears to be similar in cells from both animal species.

There are differences, however, in the magnitude of the responses to secretagogues at the two types of cells. For example, transient exposure to 60 mM K^+ induces a lower increase in free cytosolic Ca^{2+} in rat cells when compared to bovine cells, but induces a similar quantity of exocytosed CA, and for a similar duration. Activation of nicotinic receptors with DMPP leads to

longer duration of exocytosis at both cell types than that induced by exposure to K^+, even though the agents are exposed to the cell for the same period of time. This secretion is accompanied by a prolonged elevation of cytosolic Ca^{2+}.[12] However, the responses to DMPP are less prolonged in rat cells resulting in less total catecholamine release. We have previously suggested that the longer time course of release with nicotinic stimulation compared to K^+ stimulation at bovine cells is due to the hydrophobic nature of the secretagogue,[12] and this may play less of a role at rat chromaffin cells. Alternatively, since activation of nicotinic receptors have been shown to induce localized release of internal Ca^{2+} stores as well as direct cellular depolarization in bovine cells,[25] the former effect may be less effective in rat cells.

The histamine response in Ca^{2+}-containing medium is apparently via H_1 receptors whose presence has been documented on bovine[27,30] and rat[3] chromaffin cells. In the bovine chromaffin cell, histamine-induced Ca^{2+} influx is via Ca^{2+} channels that are not voltage-gated[1] and are homogeneously distributed over the cell membrane.[6] As with DMPP the Ca^{2+} elevation and secretory response due to histamine exposure are prolonged relative to K^+, and are of greater duration in the bovine chromaffin cell compared to the rat. Prolonged Ca^{2+} influx induced by histamine at bovine chromaffin cells has been previously reported and attributed to the slow desensitization of the H_1 receptors.[27] Our observations of a decline in secretion and maximal cytosolic Ca^{2+} with repetitve stimulations at rat cells provide evidence for desensitization in rat cells since subsequent K^+ exposures show robust responses.

Different sensitivity of bovine and rat chromaffin cells to internal Ca^{2+} store releasing agents. The major differences found between chromaffin cells isolated from the rat and cow are with secretagogues which activate internal stores. Chromaffin cells isolated from the cow differ from rat cells in that histamine can induce secretion in Ca^{2+} free media in the former but not the latter (Fig. 4B and 4D). The Ca^{2+} response at bovine cells in Ca^{2+}-free media differs from that in Ca^{2+}-containing media in that a more transient (~ 10 - 40 s) increase in cytosolic Ca^{2+} is observed (Fig. 4C and 4D). In the latter media this response is accompanied by a longer plateau phase of

elevated cytosolic Ca^{2+} which is interpreted to be due to the influx of external Ca^{2+}.[2,6]

Muscarinic receptors have been reported to be more important in the control of secretion at chromaffin cells from the rat[39] than the cow.[5] Our results confirm this difference at the single cell level since methacholine (50 μM), a muscarinic agonist, consistently induced both a significant increase in cytosolic Ca^{2+} and secretion in rat cells, but only did so in 30% of the bovine cells tested in the presence of extracellular Ca^{2+}. Without extracellular Ca^{2+}, methacholine occasionally induced catecholamine spikes from rat cells, supporting the concept of muscarinic mediated liberation of intracellular Ca^{2+} stores, but none were observed at bovine cells. Rat cells exposed to muscarinic agonists have been shown to preferentially secrete epinephrine[3] whereas bovine cells have 2-3 times the muscarinic receptor density on norepinephrine-containing cells.[24] Since cell cultures used in this work from both species are epinephrine-enriched, the greater response to methacholine at rat cells could be due to this enrichment.

Low concentrations (0.3 to 3 mM) of caffeine were found to be sufficient to elicit an increase in cytosolic Ca^{2+} accompanied by secretion at rat chromaffin cells, but were not at bovine cells. Caffeine concentrations necessary to cause similar responses in bovine chromaffin cells (10 or 40 mM)[12] caused the membrane of rat cells to rupture suddenly as evidenced by the rapid loss of fura-2. Cell death has often been linked to a large amount of cytosolic Ca^{2+}.[33,38] These results indicate that the caffeine-sensitive pools in rat chromaffin cells are either more sensitive or contain larger amounts of Ca^{2+} than those in bovine cells.

Bradykinin can increase cytosolic Ca^{2+} by liberating Ca^{2+} from an IP_3-sensitive internal store in bovine chromaffin cells.[28,29] Transient application of bradykinin (200 nM or 1 μM) to bovine chromaffin cells caused an increase in cytosolic Ca^{2+} with accompanying CA release as previously shown.[12] When this experiment was attempted at rat chromaffin cells, surprisingly low levels of bradykinin (~ fM, provided by diffusion from a large micropipette) were found to induce

frequent oscillations in cytosolic Ca^{2+} which were often of sufficient magnitude and duration to induce CA exocytosis. Such oscillations of intracellular Ca^{2+} in rat chromaffin cells have been recently reported to be recruited by exposure to bradykinin or high K^+.[10,23] Note that these oscillations are not due to an alteration of Ca^{2+} buffering by fura-2[43] because oscillations in secretion were also observed under the same conditions in the absence of fura-2. This electrochemically-detected oscillatory secretion is unique because the measurement is devoid of any perturbation of the cell membrane or potential intracellular buffering. We have previously shown that veratridine can also induce oscillations of secretion and cytosolic Ca^{2+} through the entry of external Ca^{2+} at bovine chromaffin cells.[12] Contrary to previous reports on the rat chromaffin cell,[23] spontaneous Ca^{2+} transients were never seen in resting rat or bovine chromaffin cells. Culture conditions and duration can affect the nature of oscillatory responses[13] which could explain this discrepancy (Wakade, A.R., personal communication).

Interestingly, reports have indicated that the Ca^{2+} oscillations in rat chromaffin cells induced by bradykinin are generated via an alternative mechanism, the mobilization of a caffeine-sensitive internal Ca^{2+} store.[23] Our finding that caffeine-releasable Ca^{2+} stores are much more sensitive in rat than bovine chromaffin cells is consistent with this view. Indeed, bovine chromaffin cells did not exhibit bradykinin-induced Ca^{2+} oscillations even upon exposure to concentrations > 200 nM. Oscillatory responses in rat cells to bradykinin were not found with external media that was Ca^{2+}-free. Thus, if mobilization of an internal store occurs and leads to the oscillitory responses, it may be due to Ca^{2+}-induced Ca^{2+}-release. A depletion and replenishing of the internal Ca^{2+} stores as a result of exposure to bradykinin could lead to the oscillations in cytosolic Ca^{2+} and CA secretion observed in rat chromaffin cells.

CONCLUSIONS

Secretion responses were found to be quite similar at rat and bovine chromaffin cells. Individual secretory vesicles from rat chromaffin cells were found to release similar amounts of catecholamine. Furthermore, the contents are extruded in a similar time course when compared to events at bovine cells. When a larger secretory response was elicited at bovine cells than at rat (ie. DMPP and histamine), the difference could be attributed to a prolonged elevation of cytosolic Ca^{2+} induced by the agents at bovine cells. However, intracellular Ca^{2+} store-mobilizing agents were found to have a more profound effect on maximal cytosolic Ca^{2+} and CA secretion at rat chromaffin cells than bovine. It appears that intracellular Ca^{2+} stores in rat chromaffin cells are more readily liberated than those in bovine cells, whereas some receptor-mediated stimuli have a more prolonged affect on bovine chromaffin cells. The above characterization of secretion and Ca^{2+} responses at single rat chromaffin cells lays the foundation for additional investigations of these cells. The use of laboratory animal models, such as the rat, will minimize the biological variability often present in the more commonly used bovine chromaffin cells. Hypertensive, diabetic and obese rats are available allowing the study of stimulus-secretion coupling under pathological conditions which involve catecholamines. Use of laboratory animals also allows *in vivo* pretreatment with various agents prior to cell isolation, allowing new areas of chromaffin cell biology to be investigated.

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TABLE 1. **Comparison of Ca^{2+}_{tot} and CA_{tot} from single rat and bovine chromaffin cells transiently exposed to agents in the presence of 2 mM extracellular Ca^{2+}.** All values represent integrated areas under Ca^{2+} and CA response curves for 60 s after secretagogue application. Values are given as a percentage of those obtained for 60 mM K^+ at that cell type (Ca^{2+}_{tot}: rat = 1340 ± 250 nM*s, bovine = 2140 ± 200 nM*s; CA_{tot}: rat = 0.99 ± 0.21 fmol, bovine = 1.11 ± 0.11 fmol). The percentages are given as the mean ± the standard error of the mean. Each number represents the average of at least 5 stimulations.

Agent	Ca^{2+}_{tot} Rat	Ca^{2+}_{tot} Bovine	CA_{tot} Rat	CA_{tot} Bovine
K^+ (60mm)	100 ± 30	100 ± 13	100 ± 30	100 ± 14
DMPP (50µM)	286 ± 34	390 ± 56	148 ± 31	249 ± 25
His (50µM)	51 ± 1.4	147 ± 11	55 ± 9.4	93 ± 22

FIGURE LEGENDS

FIG. 1. **Block diagram of instrumentation for simultaneous electrochemical measurement of catecholamine release and fluorescent detection of Ca^{2+} influx.** The block diagram depicts the instrumentation employed for fluorescence excitation (xenon arc lamp (XL), shutter (S), excitation filter wheel (FW), dichroic mirror (DM) and a Zeiss Fluar 40 x oil-immersion objective (40 X, 1.3 NA)) and that used to collect fluorescence emitted from the cell (C) (pinhole aperture (PA, 43 μm in these experiments), a 510 nm emission filter (EF) and a Hamamatsu R928 photomultiplier tuber (PMT)). Apparatus used for electrochemical measurements are found above the pictured cell--a carbon-fiber microelectrode (CFME), a sodium-saturated calomel reference electrode (RE) and an EI-400 potentiostat (E_{app} = +650 mV). The pressure-injection pipette (P) used to transiently apply secretagogues is shown to the left of the cell.

FIG. 2. **Simultaneous measurement of cytosolic Ca^{2+} and catecholamine secretion of single rat and bovine chromaffin cells induced by depolarizing stimuli.** An increase in cytosolic Ca^{2+} (dashed line, left axis) and catecholamine release spikes (solid line, scale bars at right) are induced by a 3-s delivery of different secretagogues as indicated by the arrows. (A) Rat chromaffin cell responses to 140 mM K^+. (B) Bovine chromaffin cell responses to 140 mM K^+. (C) Rat chromaffin cell responses to 50 μM DMPP. (D) Bovine chromaffin cell responses to 50 μM DMPP.

FIG. 3. **Comparison of quantal size and kinetics of release of individual catecholamine vesicles from the rat and cow.** Histograms of charge (Q) values for secretion events from rat (solid bars) and bovine (open bars) chromaffin cells exposed to 10 μM DMPP (3 s) at pH 7.4. Rat mean charge from 438 spikes at 7 cells is 1.5 ± 0.12 pC and bovine mean charge from 817 spikes at 7 cells is 1.3 ± 0.08 pC.

FIG. 4. **Histamine-induced cytosolic Ca^{2+} and catecholamine release from single rat and bovine chromaffin cells.** Single cells were transiently exposed (5 s) to 50 μM histamine as

indicated by the arrows. (A) Cytosolic Ca^{2+} (dashed line) and catecholamine secretion (solid line) from a single rat chromaffin cell in media with 2 mM Ca^{2+} (n = 5 cells). (B) Rat cell in media with 0.2 mM EGTA (n = 5 cells) (C) Cytosolic Ca^{2+} and catecholamine secretion from a single bovine chromaffin cell in media with 2 mM Ca^{2+} (n = 13 cells). (D) Bovine cell in media with 0.2 mM EGTA (n = 9 cells).

FIG. 5. **Methacholine-induced responses at rat and bovine chromaffin cells in the presence and absence of extracellular Ca^{2+}.** A 5-s delivery of 50 μM methacholine, in the presence or absence of extracellular Ca^{2+}, was given following a control of 60 mM K^+ (as indicated by the arrows). (A) Rat cell in media with 2 mM Ca^{2+} (n = 6 of 6 cells). (B) Bovine cell in media with 2 mM Ca^{2+} (n = 8 of 17 cells). Inset (n = 5 of 17). (C) Rat cell in media with 0.2 mM EGTA (n = 5 of 7 cells). (D) Bovine cell in media with 0.2 mM EGTA (n = 9 cells). Break in the trace represents 115 s.

FIG. 6. **Variety of oscillatory patterns of single rat chromaffin cells exposed to extremely low levels of bradykinin.** A large (⟩ 30 μm tip diameter) micropipette was brought in close proximity of a single rat chromaffin cell resulting in a slow leak of small amounts of bradykinin. This level of bradykinin was sufficient to cause oscillations in cytosolic Ca^{2+} and catecholamine release in rat chromaffin cells when 2 mM extracellular Ca^{2+} was present (n= 11 of 13 cells). Each panel shows an example of the type frequencies and burst durations measured.

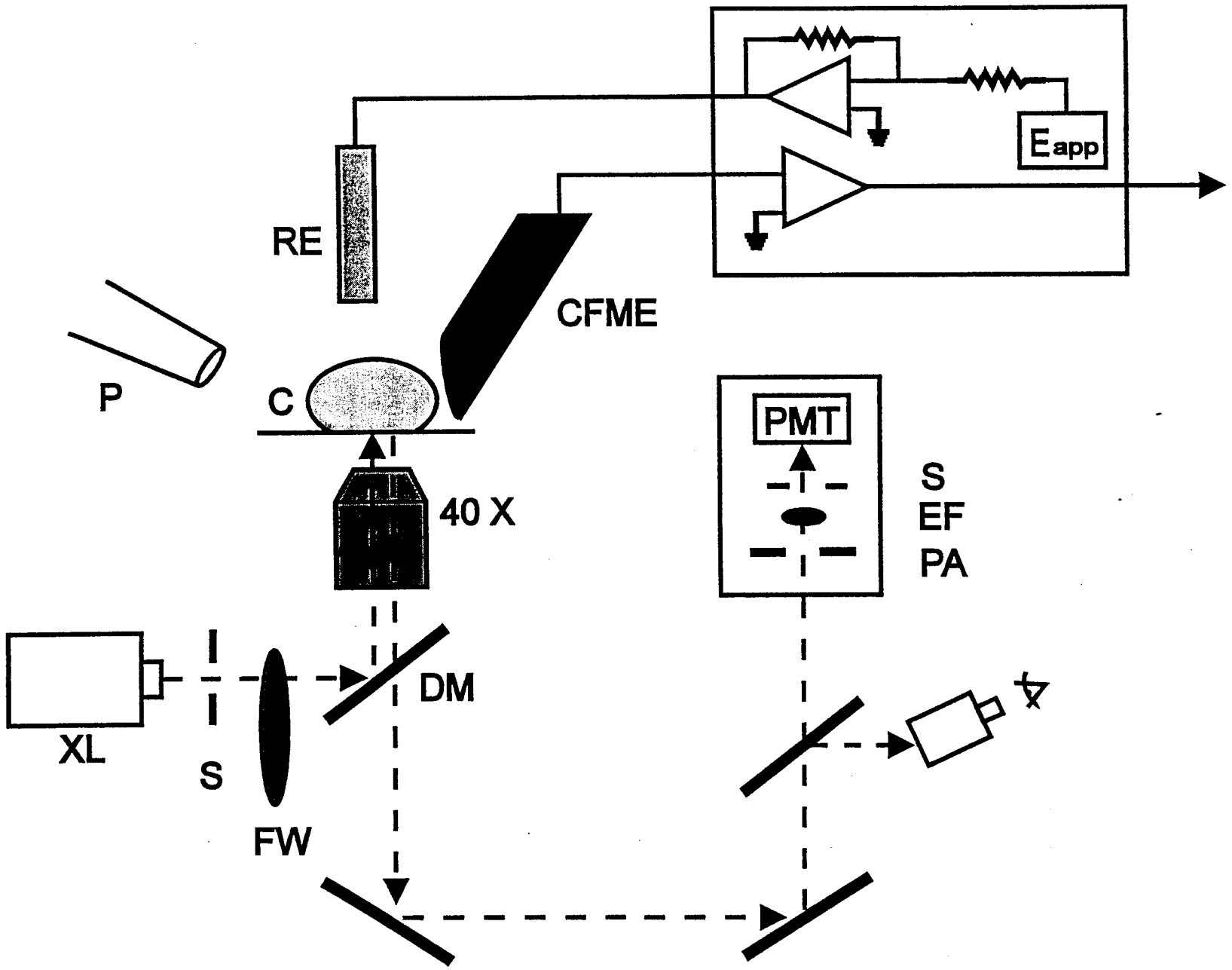

Figure 1

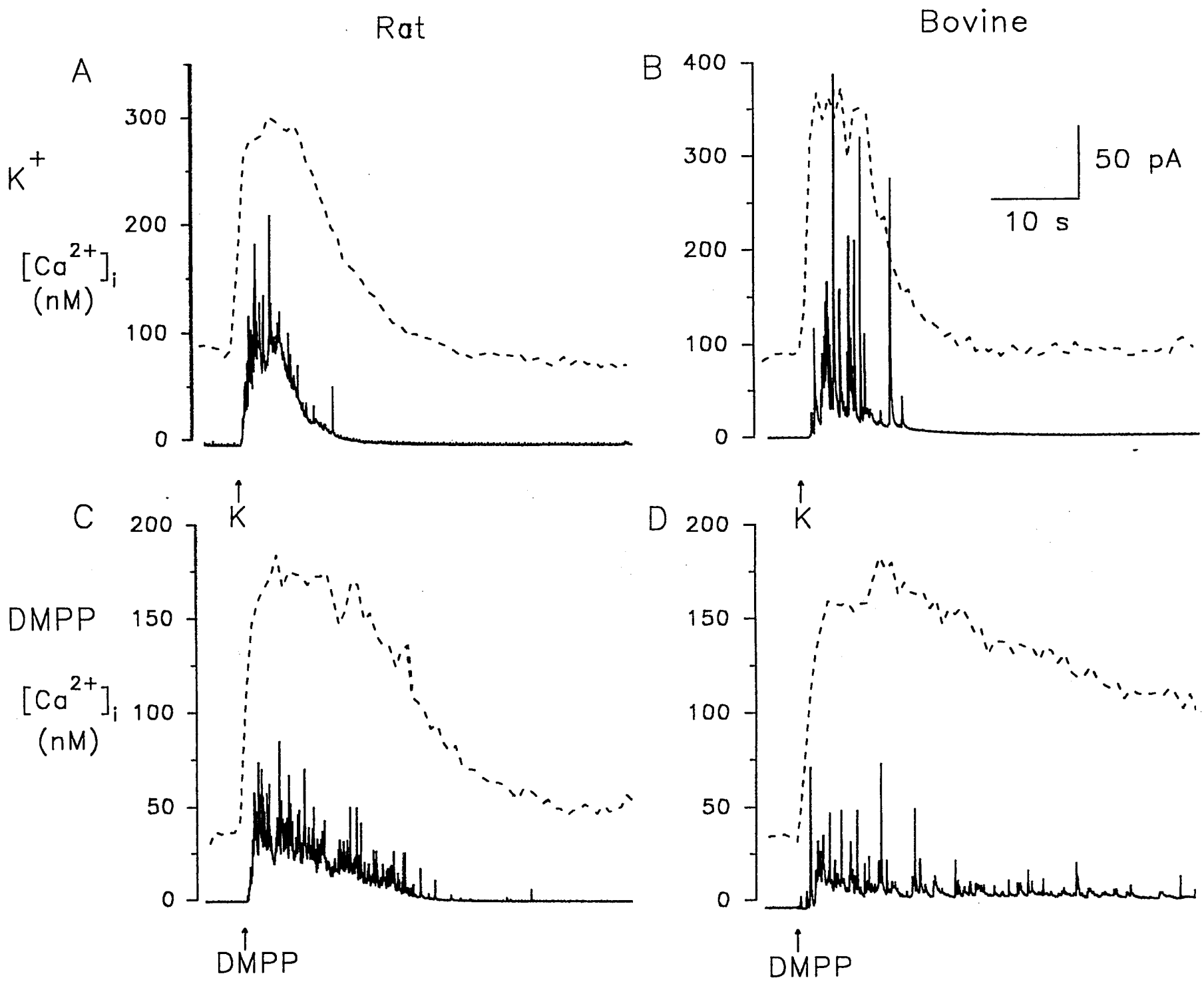
Rat
Bovine
A
B
C
D
K^+
$[Ca^{2+}]_i$
(nM)
DMPP
300
200
100
0
400
150
50
50 pA
10 s
K
DMPP

Figure 2

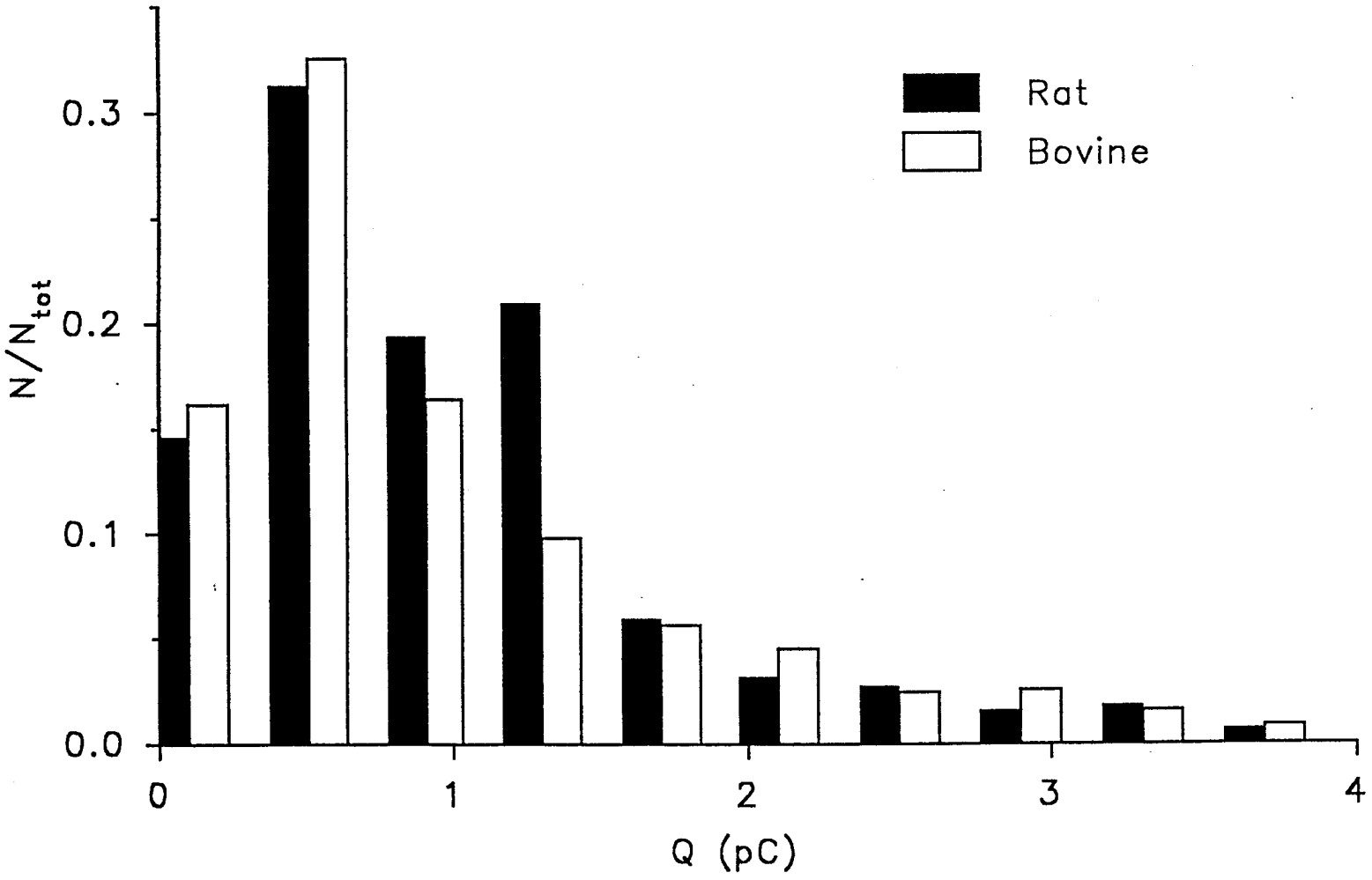

Figure 3

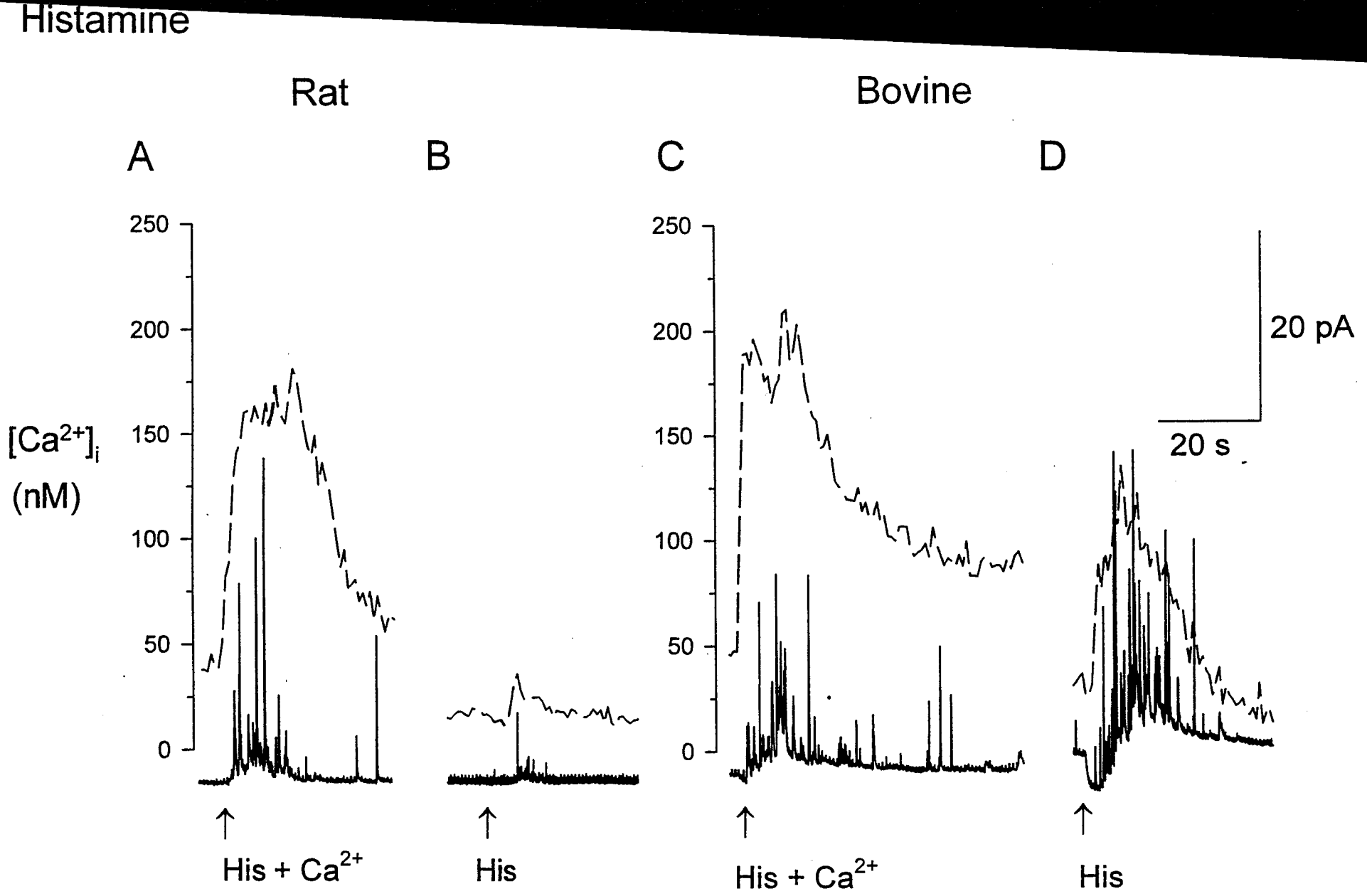
Histamine
Rat
Bovine
A
B
C
D
250
200
150
100
50
0
[Ca2+]i
(nM)
20 pA
20 s
His + Ca2+
His
His + Ca2+
His

Figure 4

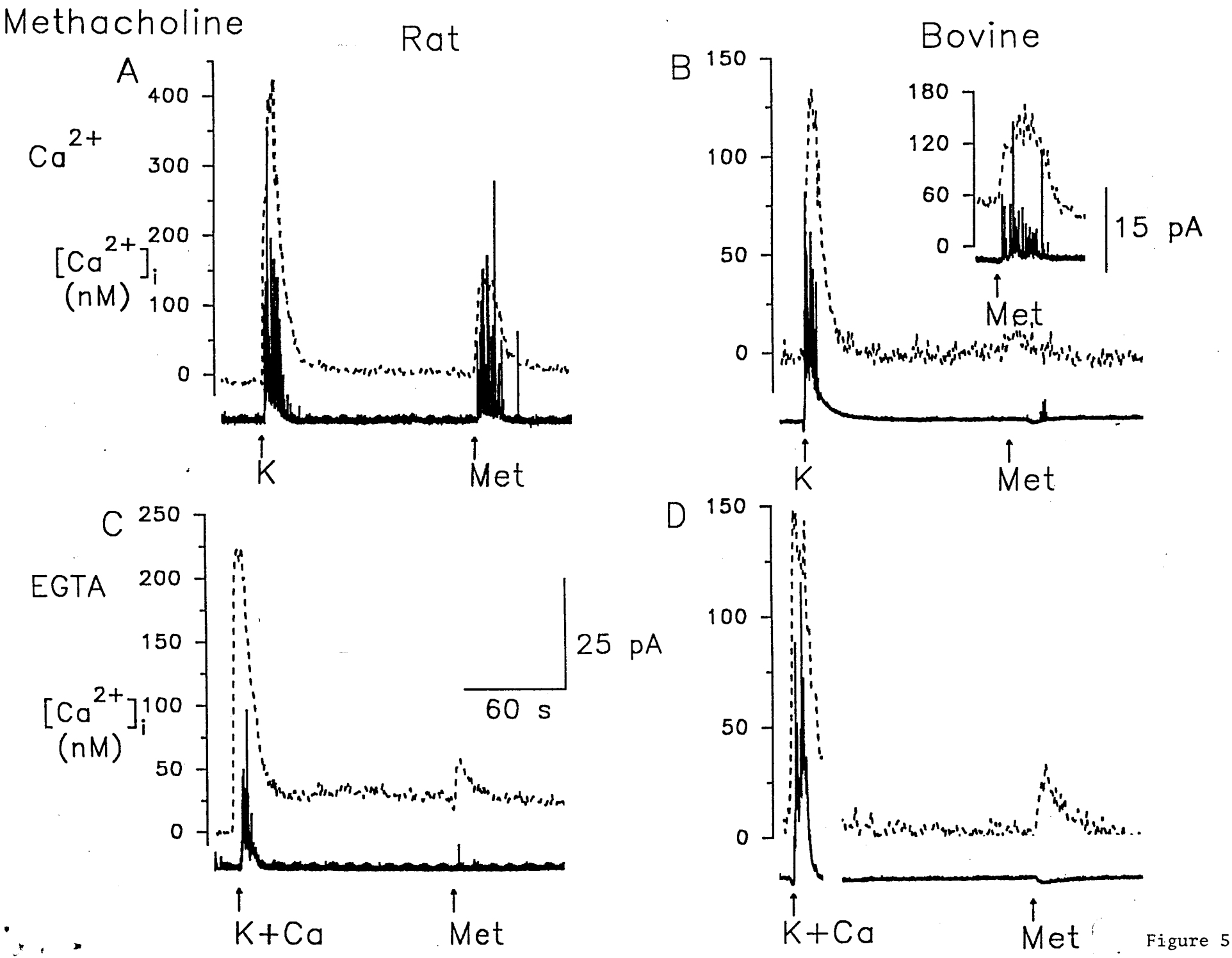
Methacholine
Rat
Bovine
A
B
C
D
Ca^{2+}
$[Ca^{2+}]_i$ (nM)
400
300
200
100
0
150
100
50
180
120
60
15 pA
K
Met
250
EGTA
25 pA
60 s
K+Ca

Figure 5

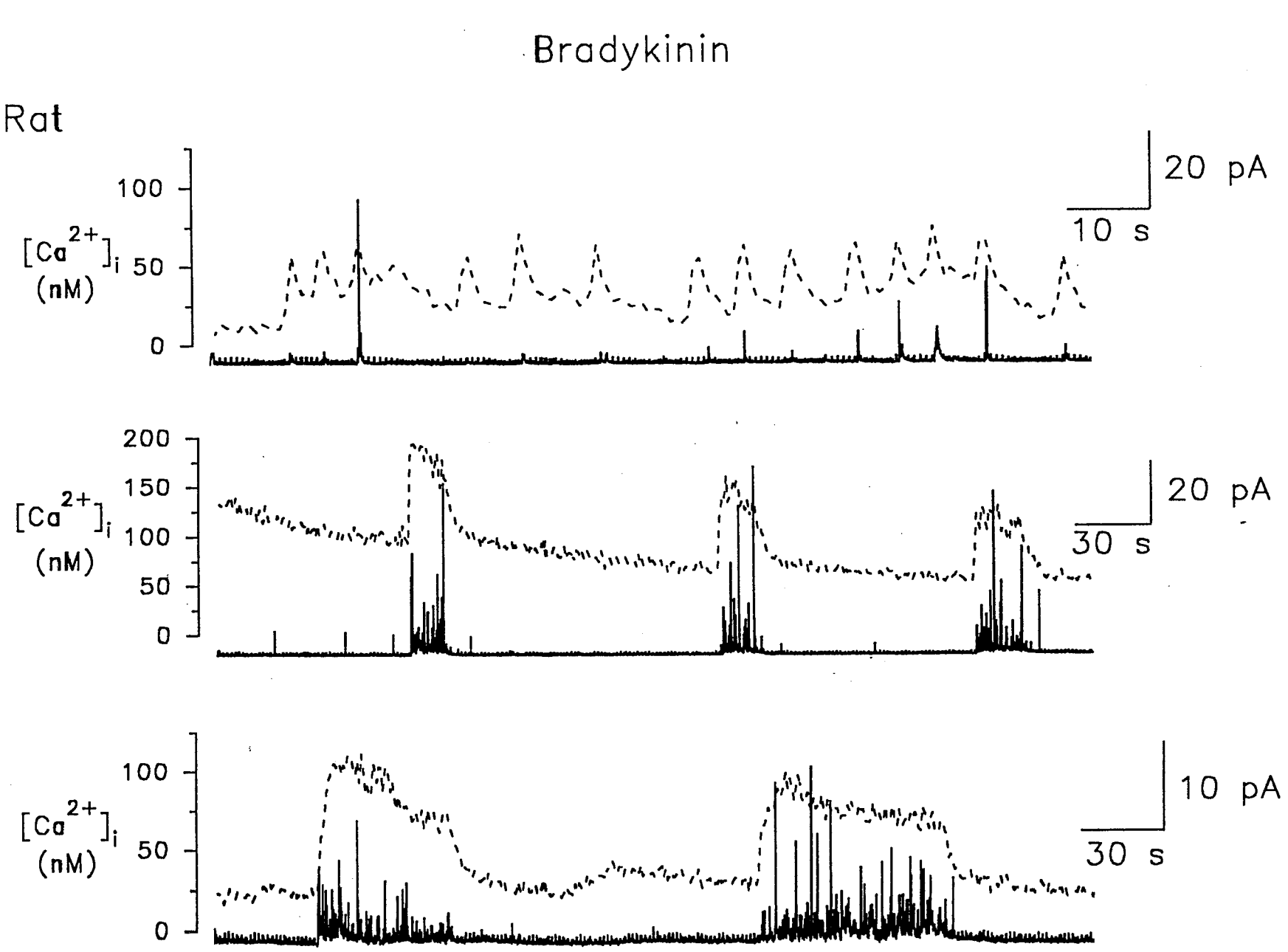
Bradykinin
Rat
100
50
0
$[Ca^{2+}]_i$
(nM)
20 pA
10 s
200
150
100
50
0
$[Ca^{2+}]_i$
(nM)
20 pA
30 s
100
50
0
$[Ca^{2+}]_i$
(nM)
10 pA
30 s

Figure 6